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The amino terminus of the Myc oncoprotein is important for many of the biological activities of the protein including transcriptional activation and repression, cellular transformation, and apoptosis. The amino terminus of Myc also contains highly conserved domains necessary for these varied functions of the oncoprotein. A yeast two-hybrid screen of a human cDNA library performed in our lab has yielded a novel factor that interacts with one of these conserved domains, Myc Homology Region II (MHR II). Analysis of this novel protein to discern potential effects on Myc function has yielded interesting results. Since MHR II is required by Myc to bring about cellular transformation, the effect of this clone on activated H-*ras* transformation was assessed. C3H10T1/2 fibroblasts were stably transfected with both activated human H-*ras* and the MHR II-associating protein, and focus formation was determined. The novel factor appears to be able to cooperate with H-*ras* to transform this cultured cell line. Another surprising potential effect on Myc function is its apparent ability to repress the transcriptional activity of Myc. Transient transfections in C3H10T1/2 cells utilizing the GAL4 reporter system and various deletion mutants of the v-Myc amino terminus have initially implicated the novel protein as a general repressor of transcription, since it not only suppresses Myc transactivation but also that of the potent activator VP16. Further analysis to assess what contribution this new factor has on Myc's other functions is underway.

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Elizabeth J Tapazowsky 6/3/96

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Myc genes encode nuclear phosphoproteins containing both a transcription activation domain (TAD) and a basic helix-loop-helix/ leucine zipper (bHLH/LZ) motif (reviewed in Henriksson and Luscher, 1996). The HLH/LZ region of Myc mediates specific protein-protein interactions between Myc and another nuclear bHLH/LZ protein, Max (Blackwood and Eisenman, 1991). Heterodimers of these Myc-Max proteins specifically bind to a hexamer DNA sequence known as an E-box (-CACGTG-) (Blackwell *et al.*, 1990). This protein oligomerization and DNA binding region is localized to the carboxy terminus of Myc.

Within the amino terminus of the Myc protein is the transcription activation domain. This region, originally identified in c-Myc by Kato *et al.* (1990), is able to activate transcription from a reporter gene driven by GAL4 protein binding sites when the c-Myc amino terminus is expressed as a fusion protein with the DNA binding domain of the yeast activator protein GAL4. The amino terminus of Myc is also necessary for many of Myc's other functions, including cellular transformation (Stone *et al.*, 1987), transcriptional repression (Li *et al.*, 1994), and apoptosis (Evan *et al.*, 1992). Contained within this region of Myc are two highly conserved domains, **M**yc **H**omology **R**egions I and II (MHRI and MHRII [Figure 1, see Appendix]). Intense efforts by our lab and others have focused on the ability of these small areas of Myc to interact with other factors. As can be interpreted from the evidence above, the amino terminus of the Myc oncoprotein is critical to several of the biological activities of Myc.

Like c-Myc, v-Myc also contains a carboxy terminal bHLH/LZ region (Min *et al.*, 1993) and an amino terminal TAD (Min and Taparowsky, 1992). v-*myc* represents the transforming sequence found in MC29, an avian leukemia retrovirus known to cause a variety of tumors (including myelocytomatosis) in infected birds (Sheiness *et al.*, 1978; Graf and Beug, 1978). Interestingly, recent evidence has suggested that certain point mutations that occur in the cellular Myc protein contribute to Myc's ability to induce cellular transformation, and some of these mutations are incorporated in v-Myc. Specifically, more than 50% of Burkitt lymphomas contain mutations in the c-Myc amino terminus at position 58, which in the wild-type protein represents a threonine residue that is phosphorylated *in vivo* (Hoang *et al.*, 1995). The corresponding site in v-Myc has been altered to a methionine residue, which, of course, cannot be modified by phosphorylation.

The implication that the viral homolog of the c-Myc oncoprotein has accumulated many of the same changes that occur in Myc-related human tumors indicates the importance

of examining v-Myc closely. Additionally, the many functions carried out by the Myc amino terminus represent how vital this region is to Myc's activity. The occurrence of mutational "hot spots" in the Myc amino terminus (Hoang *et al.*, 1995) suggests this region has a great impact on Myc's role in tumorigenesis. New studies in the field of breast cancer research aimed at determining the prognostic value of amplification of various oncogenes have demonstrated that Myc amplification (in contrast to *Her-2/neu, Int-2* amplification) was the strongest predictor for early relapse (recurrence) of the disease (Berns *et al.*, 1995). It is the goal of my research proposal to investigate how the amino terminus of Myc (specifically v-Myc) is involved in Myc's ability to induce cellular transformation.

To accomplish this goal, a detailed deletion mutagenesis of the v-Myc amino terminal TAD was performed. Based on this work and observations from other groups, the task of identifying proteins that interact with the v-Myc amino terminus was undertaken. A former postdoctoral fellow in the lab, Dr. Michael Dorsey, screened a human cDNA library using the yeast two-hybrid system to identify cellular factors that specifically associate with v-Myc MHRII. Eight independent isolates of the same clone were obtained by this screen, and the protein encoded by this cDNA (the longest isolate obtained) fails to interact with a mutant form of MHRII, indicating its specificity (M. Dorset, unpub. results). This novel protein could represent an as yet unidentified Myc-TAD-associating factor. It has been my primary goal to investigate what effect, if any, this new factor has on the biological function of Myc.

BODY

To finish characterizing the GAL4-v-Myc deletions that I initially constructed and analyzed at the beginning of my research (and described in the Annual Report covering research from May 9, 1994 -- May 8, 1995), the efficient expression of the GAL4-v-Myc fusion proteins within the culture cells used (C3H10T1/2 murine fibroblasts) was examined. Nuclear extracts were prepared from C3H10T1/2 cells transiently transfected with the various GAL4-v-Myc constructs. Despite efforts to detect the protein expression using Western analysis of these GAL4-v-Myc fusions, little success has been observed. While a positive control of 100ng of GST-GAL4 is easily detected using a monoclonal antibody against the DNA binding domain of GAL4 (obtained from Santa Cruz Biotechnology, Inc.), no detectable signals from any of the nuclear extracts tested are observable, despite the large amount of protein loaded per sample (approximately 25 μg). To circumvent this problem, I propose to use these nuclear extracts to test the DNA binding ability of the GAL4-v-Myc constructs and, indirectly, demonstrate that the various fusion proteins are being properly expressed. To accomplish this, the nuclear extracts will be incubated with a ^{32}P-labeled probe containing five GAL4 protein binding sites. The binding of each GAL4-v-Myc fusion protein will be analyzed on a polyacrylamide gel, and the intensity of the band will indicate binding strength of the GAL4-v-Myc protein. This important analytical step in the analysis of the informative GAL4-v-Myc fusions will allow determination if differences observed among the transactivation potentials of the fusion proteins are due to actual variation in TAD strength or simply due to variations in protein levels. This is especially important when comparing the 5' v-Myc deletions that have different residues at their C-termini (v-Myc residue 219 versus residue 244).

To initially characterize the interaction between the v-Myc amino terminus and the novel MHRII-associating protein identified by the yeast two-hybrid screen performed in our lab, a mammalian cell culture assay was used. Recently utilized by Kato *et al.* (1992) to demonstrate protein-protein interactions, this system allows the transactivation of a reporter gene only when two proteins associate at the reporter. Figure 2 indicates the controls developed for this assay. Briefly, the full length v-Myc protein (residues -32--416), expressed as a fusion with the DNA binding domain of GAL4 (which is unable to activate transcription of a reporter gene driven by five GAL4 protein binding sites), is allowed to interact with full length Max fused to the transcription activation domain of the Herpes simplex virus protein 16 (pVP-Max, a gift from C.V. Dang) C3H10T1/2 cells were transiently transfected by the calcium phosphate precipitation method, essentially as

described by Min & Taparowsky (1992). Precipitates used to transfect the cells contained 5 μg GAL4-v-Myc (-32--416), 5 μg of the reporter $(GAL4)_5$ E1B TATA CAT, and 10 or 25 μg of pVP-Max. Following transfection and growth in complete media for 40-50 hr., cell extracts were prepared, and equivalent amounts of extracts were assayed for the expression of the CAT protein. As can be seen in Figure 3, GAL4-v-Myc (-32--416) has no intrinsic transcriptional activity of its own; however, when pVP-Max is present in the precipitate, and steady increase in CAT activity is observed. Using this well characterized protein interaction between Myc and Max as a control, the interaction between v-Myc MHRII and the newly isolated protein (known as TRC) can be examined. TRC was also fused to the TAD of VP16 (VP16-TRC) to construct a chimeric protein. Various regions of the v-Myc TAD, fused to the DNA-binding domain of GAL4, were used to transiently transfect C3H10T1/2 cells along with VP16-TRC. The interaction of these two proteins is monitored on the $(GAL4)_5$ E1B TATA CAT reporter. As seen in Figure 3, a modest but reproducible increase in CAT activity is observed between v-Myc residues 107--219 (which contains MHR II) and VP16-TRC. This is in contrast to experiments performed with GAL4-MHR II and VP16-TRC, in which no increase in CAT activity is observed over GAL4-MHR II alone (Figure 3).

Despite the poor interaction measured in this mammalian two-hybrid assay, another cell culture technique was employed to determine the functional significance of the interaction between the v-Myc TAD and TRC. Transformation of C3H10T1/2 cells by the cooperation of v-*myc* and activated human H-*ras* oncogenes is well documented (Taparowsky *et al.*, 1987). To determine whether TRC has any effect on cellular transformation, stable transfections of C3H10T1/2 cells were performed. Separate calcium phosphate precipitates containing 200ng pT24 H-*ras*, pT24 plus 600 ng pMC29 (*v-myc*), or pT24 plus 5 μg *TRC* were made. Two 100mm plates of cells (5×10^5 cells per plate) were used per group. Two hours prior to addition of the precipitates, the cells were treated with 10 μl 100mM chloroquine. 5-6 hours after transfection, the cells were refed complete media which contained 10% fetal bovine serum. The day after transfection, cells were split 1:3 and maintained in reduced-serum media (5% FBS). Cells were fed every 3-4 days. After 18-20 days of growth, cells were fixed with methanol, stained with Giemsa, and focus formation was determined. The number of foci formed by the cooperation of H-*ras* and *v-myc* is considered the positive control, and this value was set at 1.00. As can be seen from Figure 4, *TRC* does appear to be able to cooperate with H-*ras* to induce the transformed phenotype in this cultured cell line. Perhaps TRC represents a protein that

augments the function of endogenous *c-myc* to cooperate with *ras* to transform cells. Figure 5 represents plates from each group in the focus formation assays. As can be clearly seen, while an increase in focus formation is apparent when *TRC* is present in addition to H-*ras*, the foci formed do not have the phenotype characteristic of H-*ras* and *v-myc*-induced foci. The foci formed due to cooperation between H-*ras* and *v-myc* are generally smaller, more compact, and fragmented in nature when compared to the foci formed by H-*ras* alone. H-*ras/TRC* foci are morphologically similar to H-*ras* foci, only greater in number. This suggests that TRC is not necessarily a new oncogene, but perhaps it is an auxilary factor utilized by Myc for its biological activities.

Finally, very recent observations of a potential effect of TRC on Myc's transactivation capabilities has been noted. Utilizing various GAL4-v-Myc deletions already analyzed in CAT assays, the effect of addition of a 2-fold molar excess of TRC on CAT activity was assessed. Preliminary data seems to indicate that TRC possesses the ability to repress transcriptional activity of a number of different activators. Specifically, both v- and c-Myc seem to be repressed by the presence of TRC in the precipitate when C3H10T1/2 cells are transiently transfected and assessed for CAT activity (Figure 6). In addition, even GAL4-VP16, and very potent activator and a non-Myc construct, appears to be repressed in the presence of TRC. While further studies are necessary to confirm/refute these latest observations, this could implicate a broader function of TRC when it is expressed in high excess in cells.

CONCLUSIONS

To finish the biochemical characterization of the GAL4-v-Myc deletions by assessing their ability to bind DNA (and therefore be expressed properly) may not be sufficient. If no binding is observed using the nuclear extracts, the next procedure I will use to try to detect the GAL4-v-Myc constructs is immunoprecipitation. For this experiment, COS1 cells will be transiently transfected with 5 μg of each GAL4-v-Myc plasmid. Forty hours after transfection, cells will be radioactively labeled with ^{35}S-methionine. Extracts will then be isolated from the labeled cells. A monoclonal antibody against the GAL4 DNA binding domain will be used to select the GAL4-v-Myc fusion proteins. The immunoprecipitates will be visualized by electrophoresis on an SDS-polyacrylamide gel and exposure to film. The levels of each protein will indicate if the differences seen in transactivation assays are due to differences in protein expression.

Further analysis to determine the funcion of TRC will be performed. The differences seen in Figure 3 between GAL4-v-Myc (107-219) and GAL4-v-Myc-MHR II could be explained by the absence of sufficient amounts of Myc for TRC to interact within this type of mammalian two-hybrid assay. Since the interaction of two well-known dimerizing proteins, Myc and Max, results in only a 4.5 fold increase in CAT activity over full length Myc alone in a similar assay (Figure 2), the two-fold increase in activity seen with GAL4-v-Myc (107-219) and VP16-TRC may be significant. An experiment to subclone a mutant form of MHR II in the context of v-Myc residues 107-219 is underway, and failure of this GAL4-v-Myc mutant to be active in the presence of TRC will indicate the specificity of this interaction.

Additionally, both the CAT assay of Figure 3 and the focus assays of Figures 4 and 5 have been performed with only a partial clone of TRC. A newly isolated full length clone (obtained by Dr. Michael Dorsey) will be used to repeat these experiments. This clone will be transferred into a mammalian expression vector, such as the pDCR vector. This will allow the expression of this clone in our cultured cells, and will also allow the detection of this protein using an hemagluttinin (HA) antibody. Stable transfections of C3H10T1/2 cells with the full length clone will be performed, and cooperation with activated H-*ras* will be analyzed as described previously.

Besides repeating the stable transfections with the full length TRC clone, this clone will be transferred into the pBluescript vector so it will be able to be *in vitro* transcribed and translated, and this protein will be used in bandshift assays. Nuclear extracts from transiently transfected C3H10T1/2 cells will be harvested. These extracts will contain

various GAL4-c-Myc constructs obtained from M. Cole. These constructs consist of the full length c-Myc TAD (residues 1-262), the c-Myc TAD minus MHR II, and the c-Myc TAD with two point mutations within MHR II (W136E and W136G) (Brough *et al.*, 1995). These extracts will be incubated with a labeled GAL4 probe and run out on a polyacrylamide gel. Supershifts using *in vitro* translated TRC will then be performed. Interaction of TRC with the c-Myc TAD mutations will help to define the specificity of this interaction. Similar experiments will be performed with v-Myc.

Next, since MHR II is necessary for Myc to induce apoptosis, the potential role of TRC in Myc-mediated apoptosis will be investigated. Rat-1 cells expressing *c-myc* constitutively undergo apoptosis when serum-starved (growth in media with 0.1% serum), and this phenomenon is dependent upon MHR II (Evan *et al.*, 1992). In order to address the question of whether or not TRC has a funcion in apoptosis, a clone of C3H10T1/2 cells expressing TRC will be isolated, and its growth properties in both growth- and low-serum media will be analyzed. If TRC does participate in apoptosis, cells maintained in low-serum media should begin to undergo apoptosis. Conversely, TRC could perhaps protect cells expressing Myc from undergoing apoptosis, especially if a role in cellular transformation has been implicated. To examine this possibility, a v-Myc cell line, generated and characterized in our lab (Myc neo 13A) will be stably transfected with TRC. Myc neo 13A cells and Myc neo 13A plus TRC cells will then be grown in low-serum media. The v-Myc cell line will undergo apoptosis upon serum withdrawal. The effect of TRC on v-Myc-mediated apoptosis will be determined.

Finally, the apparent non-specificity of the observed "repressor" function of TRC on transcriptional activation (Figure 6) will be more thoroughly analyzed. The c-Myc deletions from M. Cole will be utilized to determine if mutations in MHR II have any effect on TRC repression. Also, other activators, such as the bHLH myogenic protein MRF4 will be tested on an appropriate reporter in the presence of TRC. This should help determine if this supposed repressor function of TRC is due to a general mechanism applicable to any type of activation assay.

With the isolation of TRC, one of the overall goals of this research proposal has been realized, namely to use the basic information generated from a detailed analysis of the v-Myc amino terminus to search for other cellular factors that could be important to Myc's biological activities. While the initial data gathered during the examination of TRC seems puzzling at times, it is the plan of this researcher to pursue this novel interaction of TRC and v-Myc to hopefully elucidate some aspect of Myc function via association of this new factor.

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APPENDIX

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                          1
PDIQQLIRAAPSTVHGQAAAAAMPLSASLPSKNYDYDYDSVQPYFYFEEEEENFYLAAQQ     v-Myc
**********************MPLNVSFTNRNYDLDYDSVQPYFYCDEEE*NFY**QQQ     c-Myc
*****M********PG************MICKNPDLEFDSLQPCFY**PDEDDFYFGGPD     N-Myc
*********************************MDYDSYQHYFYDYDCGEDFY*****       L-Myc

              MHR I
RGSELQPPAPSEDIWKKFELLPMPPLSPSRRSSLAAASC*****FP************ST     v-Myc
QQSELQPPAPSEDIWKKFELLPTPPLSPSRRSGLCSPSYVAVTPFSLRGDNDGGGGSFST     c-Myc
******STPPGEDIWKKFELLPTPPLSPSR*************GFA****EHSSEPPSWV     N-Myc
*****RSTAPSEDIWKKFELVPSPPTSPPW**GLGPGA**********GDPAPGIGP***     L-Myc

                                              MHR II
ADQLEMVTELLGGDMVNQSFICDPDDESFVKS***IIIQDCMWSGFSAAAKLEKVVSEKL     v-Myc
ADQLEMVTELLGGDMVNQSFICDPDDETFIKN***IIIQDCMWSGFSAAAKL***VSEKL     c-Myc
TEMLL*ENELWGSPAEEDA*FGLGGLGGLTPN**PVILQDCMWSGFSAREKLERAVSEKL     N-Myc
*******PEPWPGGCTGDEAESRGHSKGWGRNYASIIRRDCMWSGFSARERLERAVSDRL     L-Myc


A*TYQASRREGGPAAASRPGPPPSGPPPP*PAGPAASAGLYLHDLGAAAADCIDPSVVFP     v-Myc
A*SYQAARKDSGSPNPARG************HSVCSTSSLYLQDLSAAASECIDPSVVFP     c-Myc
QHGRGPPTAGSTAQSPGAGAASPAGRGHGGAAGAGRAGAALPAELAHPAAECVDPAVVFP     N-Myc
APG**APRGNPPKASAAPDCTPSLEAGNPAPAA**************************P     L-Myc

                                                  219
YPLSERAPR**********************AAPPGANPAALLGVDTPPTTSSD*****     v-Myc
YPLNDSSSPKSCASQDSSAFSPSSDSLLSSTESSPQGSPEPLVLHEETPPTTSSD*****     c-Myc
FPVNKREPAPVPAAPASAP*AAGPAVASGAGIAAPAGAPGVAPPRPGGRQTSGGDHKALS     N-Myc
CPLGEPKTQACSGSESPS*****************************************     L-Myc

                                        244
*************SEEEQEEDEEIDVVTL
*************SEEEQEDEEEIDVVSV
TSGEDTLSDSDDEDDEEEDEEEEIDVVTV
******************DSENEEIDVVTV
```

Figure 1. Amino acid sequences of the amino termini of the Myc family of oncoproteins. Conserved domains are indicated. Numbering reflects residue positions in v-Myc.

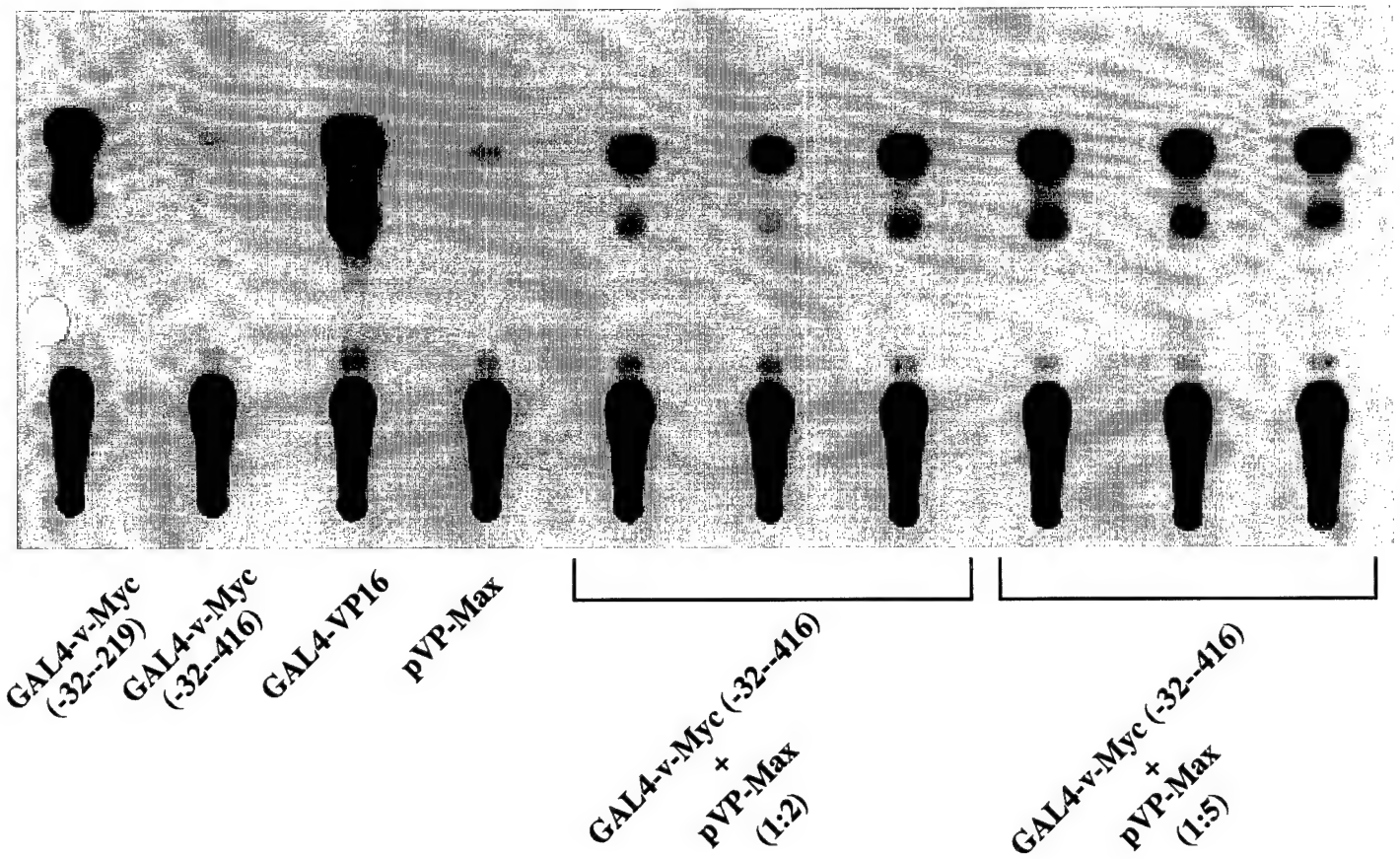

Figure 2. Transcriptional activation properties of mammalian two hybrid assay. pVP-Max was used at three- and five-fold excess over GAL4-v-Myc (-32--416).

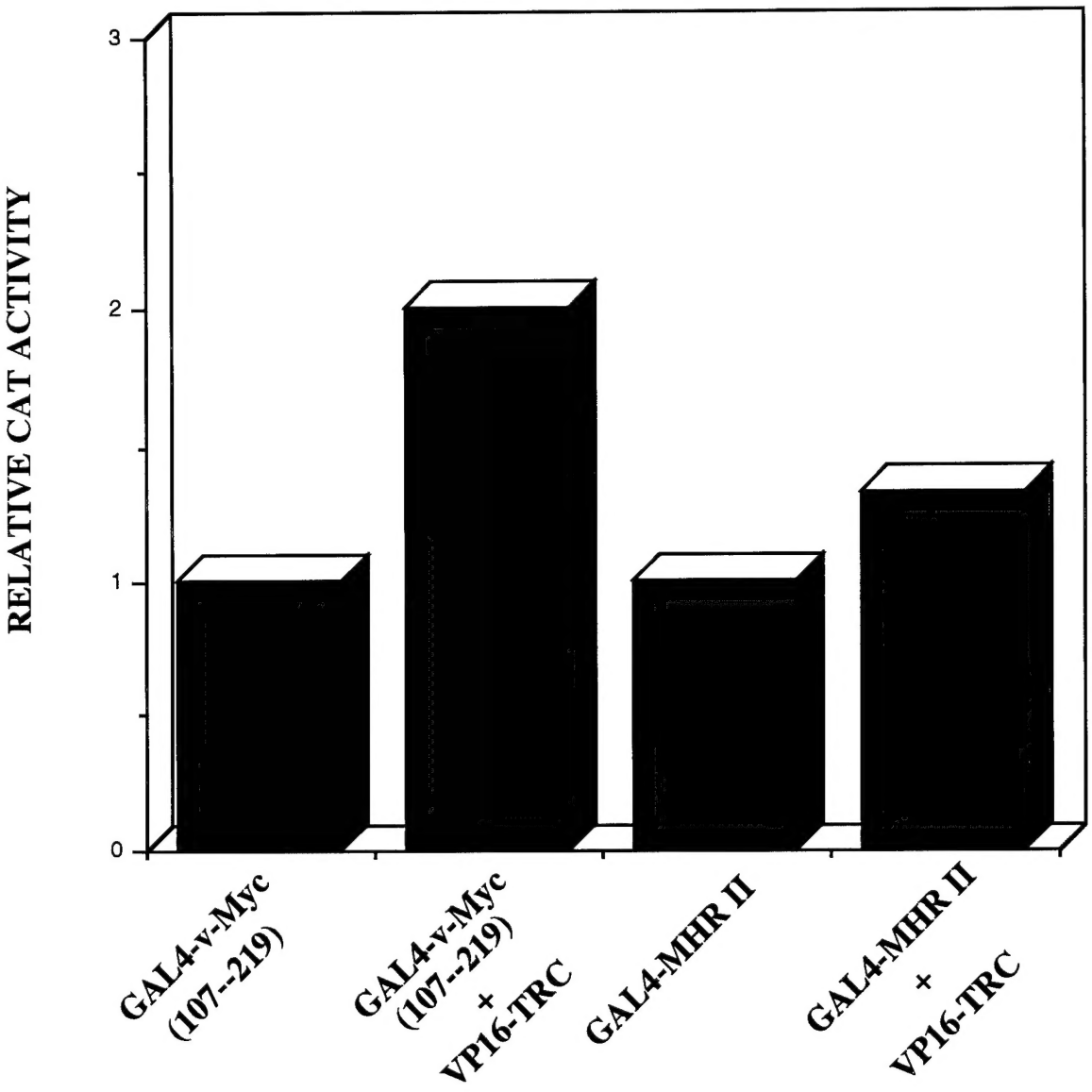

Figure 3. Transcriptional activity resulting from interaction of two proteins at the (GAL4)5 E1B TATA CAT reporter. Activity of the GAL4-v-Myc fusions is set at 1, and any increase in CAT activity due to interaction with VP16-TRC is expressed as fold over the GAL4-v-Myc fusion. Standard deviation in both experiments is 0.2.

Construct	Efficiency of Focus Formation
ras	**0.31**
ras + v-myc	**1.00**
ras + TRC	**0.94**

Figure 4. Efficiency of focus formation of H-*ras* plus *v-myc* and H-*ras* plus *TRC*. The number of foci formed by cooperation of H-*ras* and *v-myc* is set at 1.00.

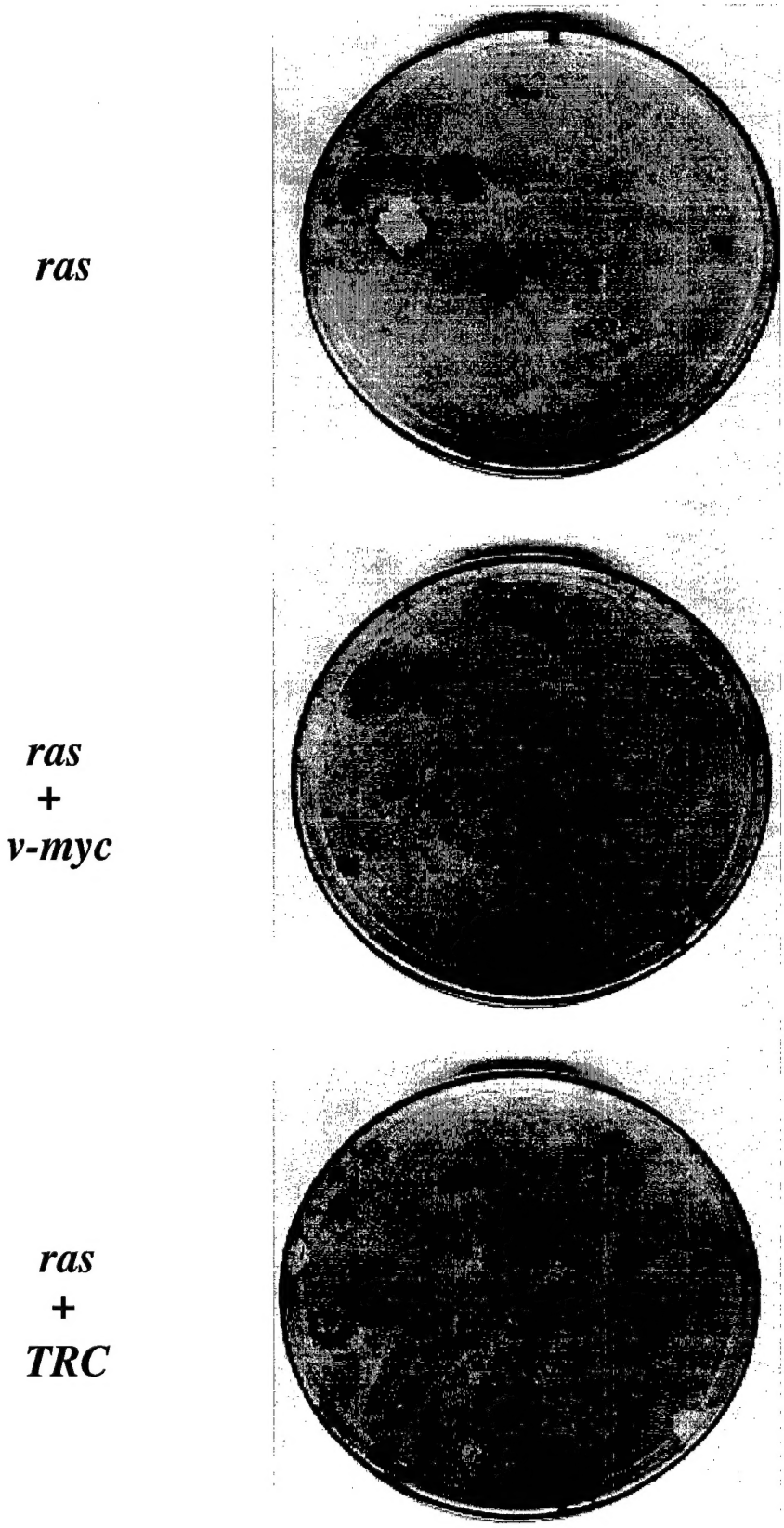

Figure 5. Focus formation assay in C3H10T1/2 cells.

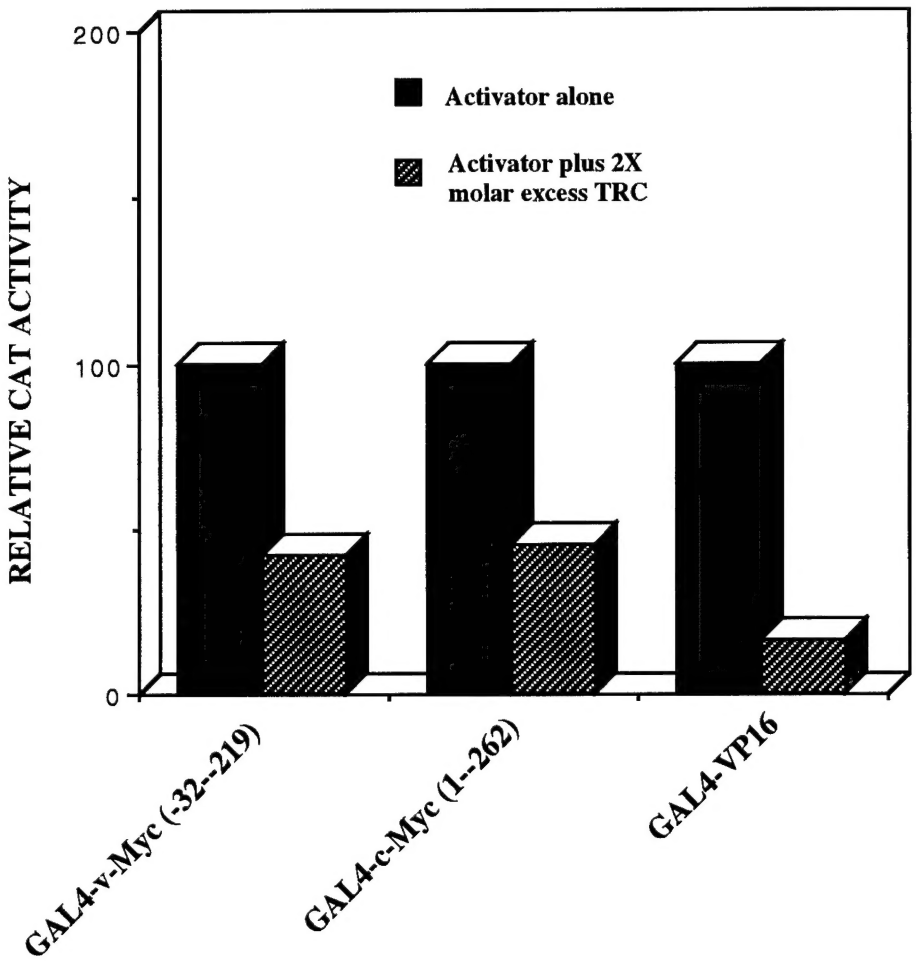

Figure 6. Transcriptional activation properties of various GAL4 activators alone and in the presence of a 2-fold molar excess of TRC.